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The recent discovery of binding protein dependent secondary transporters and the ever-growing family of membrane potential generating secondary transporters emphasize the diversity of transport systems in both the mechanistical and physiological sense. The vast amount of data on the lactose permease is now beginning to crystallize in a model that relates functional events to structural changes of the protein. Evidence has been presented that multidrug transporters pick up their substrates from the membrane, and the binding of a number of substrates to the binding-protein components of ATP-driven transporters is now understood in detail.

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Abbreviations

pmf	proton motive force
SMR	small multidrug resistance

Introduction

Transport of solutes in and out of cells is catalyzed by transport proteins that are embedded in the cell membrane. In bacteria, transporters are involved in processes as diverse as uptake of growth substrates, excretion of metabolic end-products, detoxification, metabolic energy generation and pH homeostasis. In most cases the transporters can transport the substrates against a concentration gradient, which requires the input of some energy source. Primary solute transporters use the free energy that is released upon the hydrolysis of ATP; secondary solute transporters use the free energy that is stored in the electrochemical gradients of protons and/or sodium ions across the membrane. The different types of coupling are reflected in the different architectures of the two classes of transporters. In this review we focus on recent insights into the diversity of the transport systems and the mechanism of transport and energy coupling.

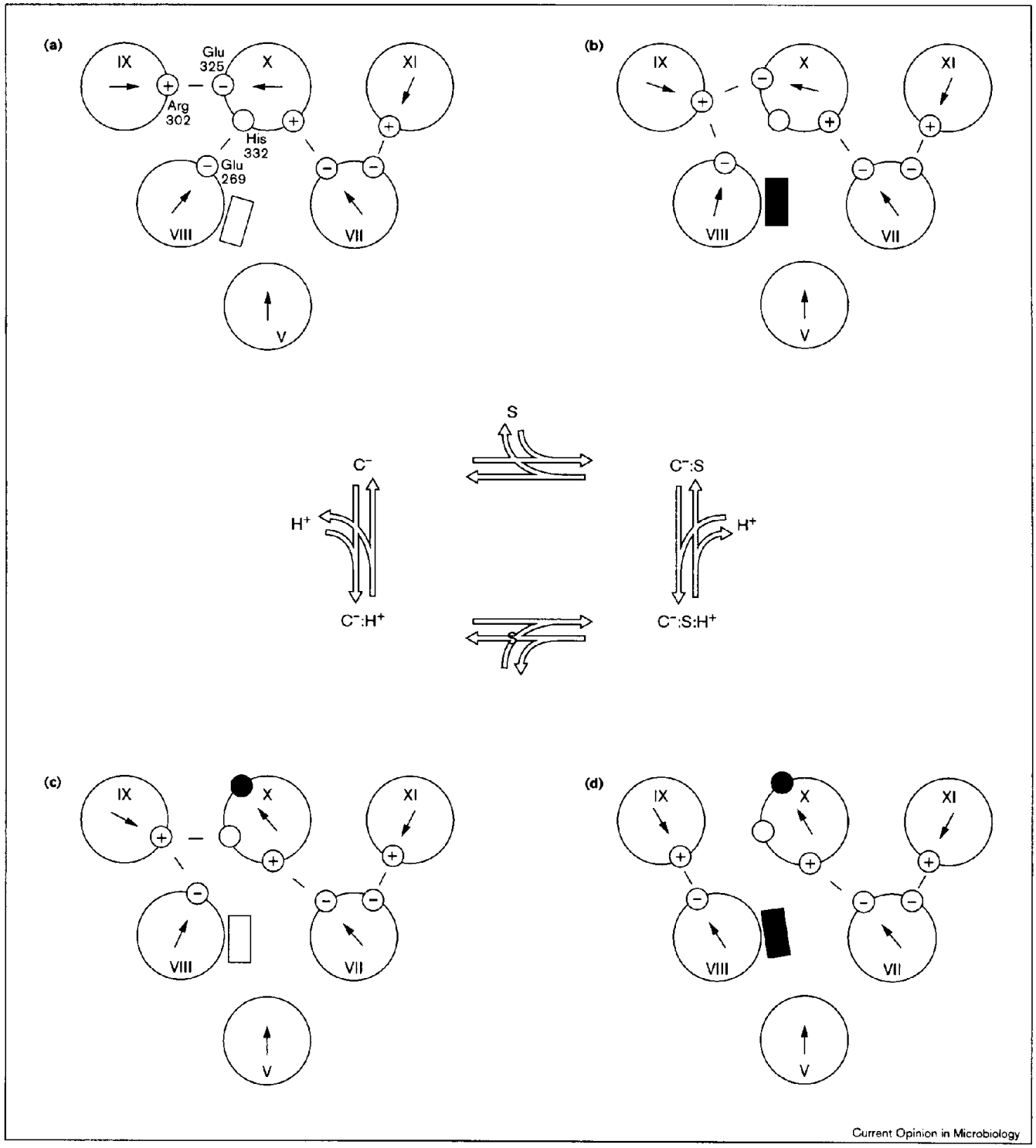
Binding protein-dependent secondary transporters

ATP-driven uptake systems are usually dependent on a binding protein that is located in the periplasm (Gram-negative bacteria) or attached to the outer surface of the cytoplasmic membrane (Gram-positive bacteria). For a long time it was believed that these binding proteins were unique to ATP-driven systems, but now it is clear that they may also form part of uptake systems driven by ion gradients. For instance, glutamate uptake in membrane vesicles of *Rhodobacter sphaeroides* is dependent on Na^+ ions, a proton gradient (proton motive force, pmf) and a glutamate-binding protein [1••]. These uptake systems are known as TRAP (for tripartite ATP-independent periplasmic) transporter. The structural features of the TRAP transporters are different from those of both ATP-driven systems and secondary transporters that do not use periplasmic binding proteins. In addition to the 12 transmembrane segments usually observed for secondary transporters, the TRAP transporter have four extra transmembrane segments, either as part of the same polypeptide or as two separate subunit [2•,3•].

H^+/solute symporters

Most of our knowledge of secondary transporters stems from work on the lactose permease LacY of *Escherichia coli* that catalyzes the coupled translocation of a solute, the β-galactosides, and a single proton (H^+ symport). LacY is a single polypeptide that consists of 12 transmembrane segments. Recently, structural data on the LacY protein and functional data on the wild-type LacY and numerous mutants were merged in a model that describes how conformational changes in the protein are related to the coupling of the solute and proton fluxes [4••]. Central to the mechanism of transport are six of the 12 transmembrane α helices, which interact which each other through charged or polar residues positioned at the interfaces of the helices (see Figure 1). The sugar binding site is situated between helices V and VIII and the proton binding site is a glutamate residue in helix X (Glu325), at the interface with helix IX. These two binding sites communicate through a redistribution of the interactions between the helices which result in changes in the tertiary structure of LacY, for instance, by rotation of the helices relative to one another or by changes in the tilt of the helices. In summary and somewhat simplified, catalysis is proposed to proceed as follows [4••]. In the unbound state (C^-), helix X interacts electrostatically with helix IX through the Arg302/Glu325 pair (Figure 1a), the latter being the proton binding site. Helix X also interacts with helix VIII through the Glu269/His322 pair. Binding of the substrate (C^-:S) breaks the latter interaction and Glu269 on helix VIII interferes with the interaction between helices IX and X (Figure 1b). As a consequence the proton binding site is moved to a more apolar environment which results in a pK_a shift and binding of the H^+ (Figure 1d). In the ternary complex (C^-:S:H^+) the interaction between helices IX and X is completely lost and helix X interacts with helix VII. In this configuration translocation can take place.

Figure 1



Molecular mechanism of H^+ and sugar binding by LacY of *Escherichia coli*. The center shows a kinetic scheme for the random binding of the the proton (H^+) and a sugar (S) to the transporter (C^-). The four models show the top view of the helix packing in the transporter protein. Models **(a,b,d)** are simplifications of the ones presented by Kaback [4••]. Model **(c)** is the one proposed in the text. Large circles indicate the transmembrane α helices (V, VII–XI). Small circles marked + represent arginine or lysine residues, small circles marked − represent glutamate or aspartate residues and unmarked small circles represent histidine. The proton binding site is the glutamate residue in helix X and the rectangle is the sugar binding site. Occupied binding sites are shown in black. The arrows in the helices indicate the relative orientations in the four models.

Coupling of proton and solute fluxes by a secondary transporter requires that the conformational changes that result in the reorientation of the binding sites are possible in the ternary complex (C^-:S:H^+) and the unloaded transporter (C^-), but not in the two binary complexes (C^-:S and C^-:H^+). The latter two situations do not result in transport of sugar and proton, respectively (see also [5]). One difference between the unloaded transporter and the ternary complex on the one hand and the binary complex on the other hand is the number of interactions in which helix IX is engaged. In the former two states, helix IX interacts with helix X or VIII, whereas in the binary complex helix IX interacts with both helix X and VIII. It is possible that the latter situation does not provide enough conformational flexibility to allow reorientation of the binding sites. This hypothesis implies that similar constraints exist in the binary complex of the transporter and the proton. Such a complex is likely to exist because equilibrium exchange is catalyzed by the protonated transporter (discussed in [4••]). By analogy to sugar (β-galactoside) binding we propose that protonation of Glu325 on helix X (Figure 1a) results in the breaking of the electrostatic bond between the Glu325/Arg302 pair and thus breaks the interaction between helices VIII and X. Helix X then moves such that the protonated Glu325 is in the more apolar environment, resulting in breakage of the bond between His322 and Glu269. The released Arg302 on helix IX cannot form a full electrostatic bond with Glu269 on helix VIII because the latter is not in the optimal orientation. Instead, Arg302 on helix IX interacts both with (released) His322 on helix X and Glu269 on helix VIII (Figure 1c) which results in a similar situation as in the C^-:S complex. Subsequent sugar binding (Figure 1d) breaks the Arg302/His322 interaction and establishes full Arg302/Glu269 bonding. Both pathways that lead to the ternary complex initially break the bond between His322 on helix X and Glu269 on helix VIII. The difference would be that sugar binding pushes Glu269 away in the direction of Arg302 on helix IX, whereas proton binding pulls His322 away in the direction of Arg302.

Kaback's model [4••] gives for the first time a molecular mechanism for the interactions between proton binding and solute binding by a secondary transporter. The next challenge is to develop models that describe the actual translocation steps.

Secondary transporters that generate membrane potential

Secondary transporters are usually looked at as energy consuming; translocation of the solute is coupled to the pmf or sodium ion motive force (smf), which allows the accumulation of the solute at the expense of the free energy stored in the cation gradient. In the past decade a class of secondary transporters has been described that are involved in the generation of a pmf (recently reviewed in [6]). Rather than being generated by proton pumping, the pmf is formed indirectly during the metabolic breakdown of weak acids into their end-products and this mechanism is termed secondary pmf generation. The first two systems that were described are oxalate fermentation in *Oxalobacter formigenes* [7] and malolactic fermentation in *Lactococcus lactis* [8]. The pathways involve, in addition to an uptake system, only one cytoplasmic enzyme, a decarboxylase. The transporters couple the uptake of oxalate and malate to the excretion of the decarboxylation products, formate and lactate, respectively (precursor/product exchange). The pathway is pmf generating because the transporters generate a membrane potential by exchanging divalent substrate and monovalent product (i.e. oxalate/formate and malate/lactate). In addition, the decarboxylation step generates a pH gradient by the consumption of a cytoplasmic proton. Even though the substrates, the products, and the chemistry of the two pathways are very similar, the transporters OxlT and MleP are not homologous [9•,10•].

Since the discovery of the oxalate/formate and malate/lactate systems, an ever growing number of similar systems have been described. Most recent examples are the glutamate/γ-aminobutyrate and aspartate/alanine systems in Lactobacilli [11,12]. A variation on the theme are the fermentations for malate and citrate in the moderate acidophilic wine bacterium *Leuconostoc oenos*. In these systems the uptake of the substrates (malate and citrate) is not coupled to the excretion of the end-products. Rather, the substrates are taken up by electrogenic uniport and the products leave the cell by passive diffusion [13,14]. Citrate uptake in *Leuconostoc mesenteroides* results in the generation of a membrane potential through the exchange of divalent citrate for monovalent lactate by CitP [15•,16]. In this case, the pathway that generates the end-product lactate is not a one-step decarboxylation, but involves multiple steps and the involvement of glycolysis. CitP is homologous to the malate transporter MleP.

A typical feature of the precursor/product exchangers described thus far is the ability to translocate two structurally related substrates. For CitP and MleP it was demonstrated that the transporters recognize specifically the 2-hydroxycarboxylate motif of the substrates [10•]. In fact, both transporters translocate a wide variety of 2-hydroxycarboxylates with the formula $R_1R_2COHCOO^-$. The high tolerance towards different R groups on the substrate allows the transporters to function as precursor/product exchangers under physiological conditions.

Multidrug transporters

It is now generally accepted that transporter proteins that confer resistance to a wide variety of toxic compounds are commonly found in all cellular membranes throughout nature. Multidrug transporters are found in both classes of solute transporter, those that are driven by ATP hydrolysis and those driven by ion motive forces. Of the latter class the family of small multidrug resistance (SMR or Mini

TEXANs, toxin extruding antiporters) are extraordinary because of their small size. They consist of a little over 100 amino acids residues and are predicted to contain only four transmembrane α helices (for a recent review see [17]). *In vitro* studies of EmrE, a small multidrug transporter of *E. coli*, reconstituted in proteoliposomes suggested that the functional unit consists of three monomers (i.e. 12 transmembrane segments), which is the common architecture for secondary transporters [18]. The EmrE multimer provides a potent experimental system for studying structure–function relationships because of its small monomeric unit and putative threefold symmetric structure.

An important question to be solved in the field of multidrug resistance is how a single protein can translocate structurally unrelated substrates that in most cases share only their hydrophobicity and a positive charge. Studies on drug excretion in *Lactococcus lactis* [19••,20] demonstrated that the substrates are pumped out of the membrane rather than from the cytoplasm. More precisely, the experiments showed that the drugs were removed from the inner leaflet of the membrane, a mechanism that is used by the pmf-driven LmrP as well as by the ATP-driven LmrA system [19••,20]. Hydrophobic ions are known to partition in the membrane and to accumulate at the membrane/water interface. The rate limiting step for passive diffusion across the membrane is the flip-flop of the compounds from outer to inner leaflet. In the proposed mechanism for multidrug resistance, the transporter takes advantage of the high drug concentration in the membrane relative to the cytoplasm and, in comparison with a mechanism in which the drug is removed from the outer leaflet, the catalyzed efflux rate competes with the slowest step in passive drug entrance. The consequence of this mechanism is that the cell has to tolerate a high drug concentration in the outer leaflet of the membrane. Structurally the mechanism requires that, for the pmf-driven LmrP, the pathway for the proton and the substrate translocation are distinct, at least in part. The proton pathway connects the water phase at the two sides of the membrane, while the substrate binding site has to be exposed to the phospholipid bilayer at some point during catalysis. The latter situation may be recognized in a recently presented model [21] of the tertiary structure of the membrane-embedded part of P-glycoprotein, a structural and functional homolog of the LmrA protein. In the model, the 12 transmembrane α helices are arranged in the form of a horse shoe presenting a clear opening to the lipid bilayer [21].

ATP-driven transport dependent on binding proteins

ATP-driven transporters belong to the superfamily of ATP binding cassette (ABC) proteins and have an overall architecture of two integral membrane domains, two subunits (or domains) that transduce the energy of ATP binding and hydrolysis to transport, and a binding or receptor protein. In contrast to the limited knowledge of the structure–function relationships in the membrane components of these systems, a wealth of information is available regarding the atomic structure and specificity of the receptor proteins [22••]. The high resolution structures of more than 10 of these proteins have been solved, and despite limited sequence similarity it appears that their tertiary structures are highly similar. Ligand recognition by receptor proteins is dominated by three important features. Firstly, the ligand is bound in a cleft between two distinct globular domains and is buried by a hinge-bending motion between the two domains. Secondly, hydrogen bonding interactions are critical in the binding of the ligands. Thirdly, additional specificity is obtained by stacking of aromatic amino acid residues against the faces of the sugar substrates. It seems plausible that the multitude of interactions are responsible for the high binding affinities of the binding proteins, which have K_ds that are usually two or three orders of magnitude lower than those of secondary transport proteins, such as the LacY protein discussed above. More recently, the liganded and unliganded structures of the dipeptide and oligopeptide binding proteins have been determined [23,24,25•,26]. Although the peptide binding proteins have an additional domain when compared to other binding proteins, their mode of ligand binding is very similar. Specificity for peptides is conferred largely by hydrogen bonds between the peptide backbone (in extended conformation) and the binding protein.

Recently, the surprising observation was made that liganded and unliganded histidine binding protein (HisJ) interacts with equal affinity with the membrane-bound complex that is formed by the HisQ, HisM and two HisP subunits ($HisQMP_2$) [27]. Since the binding proteins are present in excess over the membrane-bound complexes one would expect that, at low substrate concentrations, the excess of unliganded binding protein would be inhibitory. In a follow up study, however, a large excess of HisJ had no effect on the liganded-HisJ induced ATPase activity of the $HisQMP_2$ complex [28]. The authors suggest that unliganded HisJ does not inhibit because the HisJ–$HisQMP_2$ complex is long lived as compared to the rate of redistribution of histidine molecules among HisJ. Alternatively, the association/dissociation of HisJ is rapid compared to the ATPase kinetics. Whatever the kinetic basis for the lack of inhibition, it seems peculiar to have millimolar concentrations of binding protein that bind histidine with K_Ds in the nanomolar range and for which the membrane-bound complex has a binding constant in the millimolar range irrespective of whether histidine is present. It would seem that under physiological conditions histidine uptake is not readily limited by the binding of the ligand or its delivery to the membrane-bound complex. Also experimentally, there are some inconsistencies with regard to the interaction of liganded and unliganded HisJ with $HisQMP_2$. In contrast to the ATPase assays, *in vitro* studies of histidine transport indicate that unliganded

HisJ does compete efficiently with liganded HisJ [29]. The question thus arises whether the ATPase activity of the $HisQMP_2$ complex in the permeabilized membrane vesicles does reflect a relevant activity that can be equated with translocation [28].

The interaction of liganded and unliganded HisJ with the membrane-bound complex reflects the closed and open forms of the binding protein, respectively. For the maltose binding protein (MalE) of *E. coli* it has been shown that some ligands that do bind with high affinity (e.g. cyclic dextrins) are not transported and do not stimulate ATP hydrolysis by the membrane-bound complex that consists of the MalF, MalG and MalK subunits ($MalFGK_2$). In a recent series of papers, Hall *et al.* [30–32] report four main findings: two modes of ligand binding to MalE can be deduced from UV difference spectra (i.e. the open-liganded and closed-liganded states); nontransportable ligands are unable to convert the binding protein from the open to the closed configuration; the open-liganded state is unproductive (i.e. it does not stimulate the ATPase); and mutations in MalG that result in increased basal ATPase activity permit stimulation by open-liganded MalE, and the mutant MalG facilitates transport of cyclic dextrins [30–32]. These studies suggest that correct positioning of the ligand in the translocation channel by MalE is a prerequisite for transport, and that for the opening of the pathway activation of the ATPase by the binding protein is required.

Conclusion

Solute transporters can be discriminated by their mode of energy coupling, either ion motive force or ATP-driven transporters. Today we know that binding protein dependent and independent transport systems are found in both classes. Apparently, an initial step in the transport process in which the substrate is captured outside the membrane can be an advantage independent of the mode of energy coupling. The recent studies on the binding protein-dependent ATP driven histidine transport system are not particularly helpful in understanding the nature of this advantage. The data seem to indicate that, under physiological conditions, the transport components are saturated with binding protein irrespective of the presence or absence of ligand, which makes the binding protein merely a subunit that can alternate between the free and the bound state. The advantage of binding proteins has to be resolved in the future.

The major challenge in the field of secondary transport is to understand, at the molecular level, how the protein couples the fluxes of cation and solute. The model of Kaback [4••] that describes structural changes of LacY upon the binding of the substrate and the proton addresses this question directly for the first time. The most difficult part will be to understand the conformational change that represents the translocation step, what induces it and what prevents it. This may still be difficult to understand once a high resolution structure is available.

The observation that multidrug resistance transporters bind their substrates in the membrane raises an interesting question about the structure of secondary transporters. LmrP, the ion motive force driven multidrug resistance transporter of *L. lactis*, belongs to a large family of secondary transporters for sugars and carboxylates, substrates that are likely to be bound at the membrane/water interface. Since these transporters are likely to have the same global structure and molecular mechanism, the translocation pathway may be at the protein/lipid interface rather than through the protein.

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